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Vaccination with liposome-coupled glypican-3-derived epitope peptide stimulates cytotoxic T lymphocytes and inhibits GPC3-expressing tumor growth in mice

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ABSTRACT

Because therapeutic manipulation of immunity can induce tumor regression, anti-cancer immunotherapy is considered a promising treatment modality. We previously reported that glypican-3 (GPC3), an oncofetal antigen overexpressed in hepatocellular carcinoma (HCC), is a useful target for cytotoxic T lymphocyte (CTL)-mediated cancer immunotherapy, and we have performed clinical trials using the GPC3-derived peptide vaccine. Although vaccine-induced GPC3-peptide-specific CTLs were often tumor reactive *in vitro* and were correlated with overall survival, no complete response was observed. In the current study, we synthesized liposome-coupled GPC3-derived CTL epitope peptide (pGPC3-lipsome) and investigated its antitumor potential. Vaccination with pGPC3-liposome induced peptide-specific CTLs at a lower dose than conventional vaccine emulsified in incomplete Freund's adjuvant. Coupling of pGPC3 to liposomes was essential for effective priming of GPC3-specific CTLs. In addition, immunization with pGPC3-liposome inhibited GPC3-expressing tumor growth. Thus, vaccination with tumor-associated antigen-derived epitope peptides coupled to the surfaces of liposomes may be a novel therapeutic strategy for cancer.

1. Introduction

Immunotherapy has become a promising, refined strategy for tumor treatment [1–5]. Peptide, protein, and DNA vaccines and antibody therapy have been developed as antigen-specific immunotherapy. Tumor-associated antigens (TAAs) and TAA-derived peptides recognized by cytotoxic T lymphocytes (CTLs) have been discovered. We previously identified glypican-3 (GPC3) as a TAA. GPC3 is expressed in hepatocellular carcinoma (HCC), melanoma, and ovary cancer, but not in normal tissues except for the placenta and embryonic liver [6–8]. Hence, GPC3 is an ideal target molecule for antigen-specific cancer immunotherapy.

We previously identified HLA-A*24:02-restricted $GPC3_{298–306}$ EYILSLEEL peptide and HLA-A*02:01-restricted $GPC3_{144–152}$ FVGEFFTDV peptide, and H2-K^b/H2-D^b-restricted murine $GPC3_{127–136}$ AMFKNNYPSL as CTL epitopes [9–11]. In our clinical trial, the peptide vaccine was prepared by emulsifying GPC3

Abbreviations: CTL, cytotoxic T lymphocyte; GPC3, Glypican-3; HCC, hepatocellular carcinoma; pGPC3, GPC3-derived CTL epitope peptide; TAA, tumor-associated antigen; IFA, incomplete Freund's adjuvant; MHC, major histocompatibility complex; APC, antigen-presenting cell; OVA, ovalbumin; pGPC3-liposome, glypican-3-derived epitope peptide with liposome; B6, C57BL/6; A2-Tg mouse, HLA-A*02:01 transgenic mouse; SPF, specific pathogen-free; TAP, transporter-associated antigen processing; FBS, fetal bovine serum; pGPC3(A2), HLA-A*02:01-restricted GPC3-derived CTL epitope peptide; pGPC3(B6), H2-Kb or H2-Db-restricted murine GPC3-derived CTL epitope peptide; DMSO, dimethyl sulfoxide; CpG ODN, CpG oligodeoxynucleotide; TLR9, toll-like receptor 9; BM-DC, bone marrow-derived dendritic cell; mGM-CSF, murine granulocyte macrophage colony-stimulating factor; ELISpot assay, enzyme-linked immunospot assay; pGPC3(A2)-liposome/CpG, pGPC3(A2)-liposome containing CpG ODN; pGPC3(B6)-liposome/CpG, pGPC3(B6)-coupled liposome containing CpG ODN.

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peptide in incomplete Freund's adjuvant (IFA) and administrated by intradermal injection. However, tumor responses were observed in a limited number of patients [12–14]. In addition, it has been recently reported that IFA-based vaccination induces CTL hyporesponsiveness in mice [15]. Therefore, establishment of a new vaccine strategy is desired.

Antigen-containing liposome has been attracting interest for use as a delivery vehicle in new vaccine adjuvants. Antigens chemically coupled to the liposome surface, which consists of unsaturated fatty acids, are efficiently cross-presented via major histocompatibility complex (MHC) class I by antigen-presenting cells (APCs) and effectively stimulate antigen-specific CTLs [16]. Previously, we reported that peptide-specific CTLs were induced in mice immunized with liposome-coupled viral antigen-derived peptide, protecting the mice from viral infection [17]. Furthermore, ovalbumin (OVA)-expressing tumor regressed in mice immunized with liposomal OVA peptide [18]. In addition, liposomal peptide has the ability to induce long-lived memory CTLs in mice. The above findings on the basis of the use of a viral or model antigen indicated that liposome-coupled TAA-derived CTL epitope peptide may be promising for tumor treatment. To date, however, the therapeutic potential of such vaccine has not been clarified.

In this study, we chemically coupled GPC3-derived epitope peptide (pGPC3) to liposome surfaces (pGPC3-liposome), and investigated its ability to induce peptide-specific CTLs and its antitumor effect *in vivo*.

2. Materials and methods

2.1. Mice

C57BL/6 (B6) mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). HLA-A*02:01 transgenic mice (A2-Tg mice)—H-2D^b β2m double knockout mice transformed with human β2m-HLA-A2.1 (α1α2)-H-2Db (α3 transmembrane cytoplasmic) monochain construct—were generated in the Department SIDA-Retrovirus, Unité d' Immunité Cellulaire Antivirale, Institut Pasteur, France and kindly provided by Dr. F.A. Lemonnier [19,20]. The mice were maintained under the institutional guidelines set by the Animal Research Committee of the National Cancer Center Hospital East, Japan. The animals were housed in specific pathogen-free (SPF) conditions with a 12-h light cycle and access to food and water *ad libitum*. Six-to ten-week-old mice were used in all experiments.

2.2. Cell lines

RMA-S is a transporter-associated antigen processing (TAP)-defective B6 thymoma cell line; this cell cannot present endogenous antigens via MHC class I [21]. RMA-S-HHD is RMA-S transfected with the HLA-A*02:01-Db-β2-microglobulin single chain gene. RMA-HHD-GPC3 is RMA-HHD transfected with the murine GPC3 gene. All cells were cultured in RPMI 1640 (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), penicillin-streptomycin solution (Thermo Fisher Scientific), and 2-mercaptoethanol (2-ME, 50 μM).

2.3. Peptides

Amino acid sequences of HLA-A*02:01-restricted GPC3-derived CTL epitope peptide (pGPC3(A2)), and H2-K^b- or H2-D^b-restricted murine GPC3-derived CTL epitope peptide (pGPC3(B6)) are as follows: pGPC3(A2), FVGEFFTDV; pGPC3(B6), AMFKNNYPSL. Synthetic pGPC3(A2) was purchased from the American Peptide Company Inc. (Sunnyvale, CA, USA) and dissolved in 7% NaHCO3 solution (Otsuka Pharmaceutical Factory Inc., Tokyo, Japan) at 10 mg/mL. Synthetic pGPC3(B6) was purchased from Scrum Inc. (Tokyo, Japan) and dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries Ltd., Osaka, Japan) at 10 mg/mL.

2.4. Liposomes

"Oleoyl" liposomes, consisting of dioleoyl phosphatidyl choline, dioleoyl phosphatidyl ethanolamine, dioleoyl phosphatidyl glycerol acid, and cholesterol in a 4:3:2:7 M ratio, were used in this study.

2.5. CpG oligodeoxynucleotide

CpG oligodeoxynucleotide (CpG ODN) 5002: (5′-TCCATGACGTTCTTGATGTT-3′) was synthesized by Hokkaido System Science Co., Ltd. (Hokkaido, Japan) and was phosphorothioate-protected to avoid nuclease-dependent degradation. CpG was dissolved in distilled water at a concentration of 4 mg/mL.

2.6. Coupling of GPC3-derived peptides to the liposomes

Peptide-conjugated liposomes were prepared as described previously [22]. Liposomal peptide density was assessed by quantitative amino acid analysis (by Shimadzu Techno-Research Inc., Kyoto, Japan).

2.7. Liposomal and IFA-emulsified peptide vaccination

The liposomal vaccines pGPC3(A2)-liposome and pGPC3(B6)-liposome were administrated at 1 μg, 10 μg, or 50 μg of peptide per mouse in the presence of CpG ODN 5002 (5 μg/mouse). IFA-emulsified peptide vaccine was administered as an emulsion of peptide solution (100 μL/mouse) and IFA (100 μL/mouse). Mice were immunized with two separate intradermal injections at the base of the tail according to the schedules shown in Figs. 1A, D and 3A.

2.8. Generation of bone marrow-derived dendritic cells (BM-DCs)

BM-DCs were generated as described previously [11]. In brief, bone marrow cells (2×10^6) derived from B6 mice were cultured in RPMI 1640 containing FBS (10%), 2-ME (50 μM), and murine granulocyte-macrophage colony-stimulating factor (mGM-CSF, 20 ng/mL). After 7 days of culture, floating cells were collected and used as BM-DCs.

2.9. In vitro stimulation of splenocytes derived from immunized mice

Seven days after the last immunization, splenocytes were collected and CD8$^+$ splenocytes were isolated by positive magnetic cell sorting with anti-CD8 microbeads (Miltenyi Biotec K.K., Tokyo, Japan) according to the manufacturer's protocol. CD8$^+$ splenocytes were cocultured with peptide-pulsed BM-DCs for 7 days and the antigen-specific T cell frequency was determined.

2.10. IFN-γ enzyme-linked immunospot (ELISpot) assay

The frequency of IFN-γ-producing cells was detected using an ELISpot assay kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's protocol.

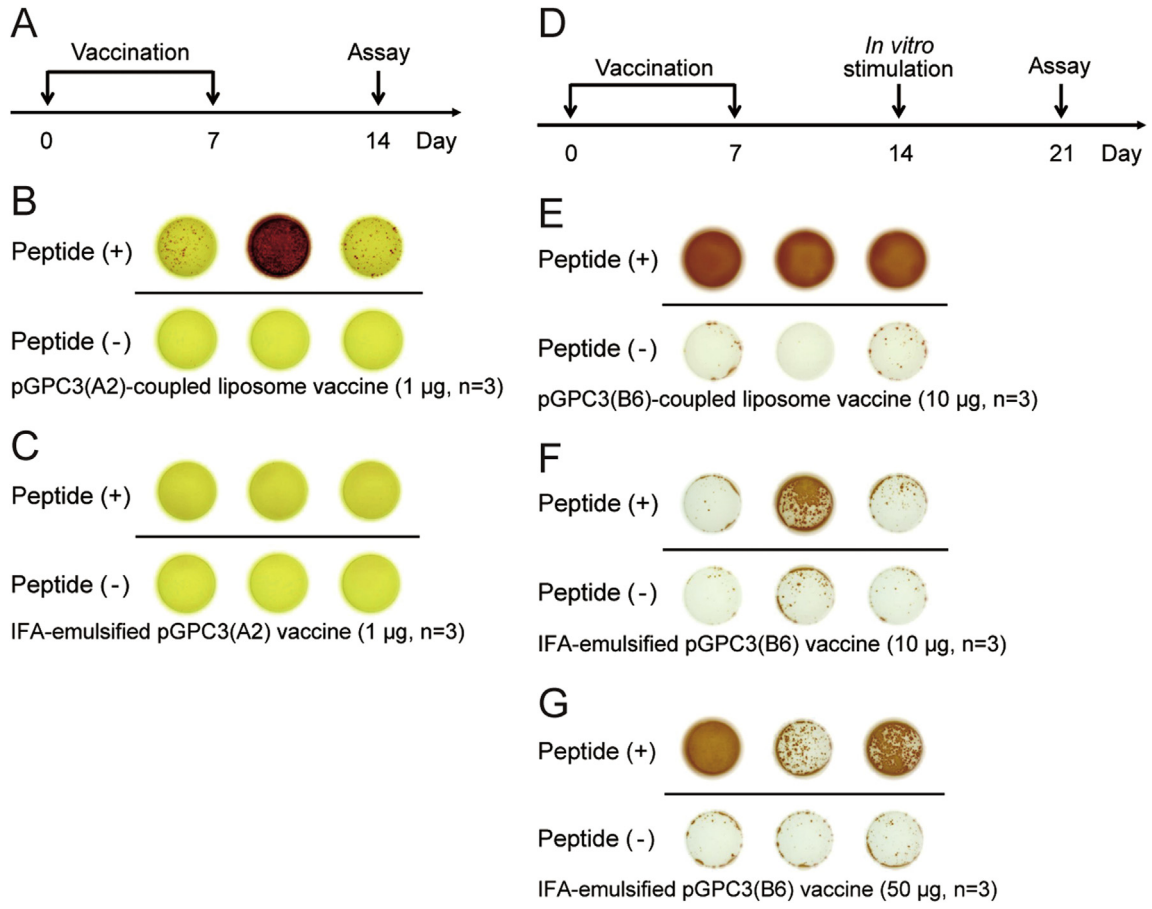

Fig. 1. Effective induction of GPC3 peptide-specific CTLs by liposome-coupled pGPC3. (A) Experimental setup for analyzing the induction of pGPC3-specific CTLs. (B, C) A2-Tg mice were immunized twice (7-day interval) with pGPC3(A2) coupled to liposomes containing CpG ODN (B) or IFA-emulsified pGPC3(A2) (C). Seven days after the second immunization, $CD8^+$ splenocytes were cocultured with RMA-S-HHD cells pre-pulsed with pGPC3(A2) for 20 h and the frequencies of IFN-γ-producing cells were evaluated by IFN-γ ELISpot assay. Peptide(+): RMA-S-HHD pulsed with pGPC3(A2), peptide(−): non-pulsed RMA-S-HHD. (D) Experimental setup for analyzing the induction of pGPC3-specific CTLs. (E, F) B6 mice were immunized with twice (7-day interval) pGPC3(B6) coupled to liposomes containing CpG ODN (E) or IFA-emulsified pGPC3(B6) (F). Seven days after the second immunization, $CD8^+$ splenocytes were stimulated with BM-DCs pre-pulsed with pGPC3(B6). After 7 days, stimulated $CD8^+$ cells were cocultured with RMA-S for 20 h and the frequencies of IFN-γ-producing cells were evaluated by IFN-γ ELISpot assay. Peptide(+): RMA-S pulsed with pGPC3(B6), peptide(−): non-pulsed RMA-S.

2.11. Tumor challenge experiment

At 14 and 7 days before tumor transfer, the mice were immunized with vaccine. At day 0, A2-Tg mice were injected with RMA-HHD-GPC3 (5.0×10^5 cells). At day 7 after tumor transfer, the mice were immunized again. The lengths of the major and minor axes of the tumor were measured by using a caliper. The tumor area was calculated according to following formula: (tumor area) = (length of major tumor axis) × (length of minor tumor axis).

2.12. Statistical analysis

Statistical analyses of the ELISpot assays and the antitumor effect of the vaccines were performed using a Mann–Whitney U or Kruskal–Wallis test. Significant differences were defined as $^*p < 0.05$.

3. Results

3.1. Liposome-coupled pGPC3 efficiently stimulates peptide-specific CTLs in vivo

We previously found that a high dose of pGPC3 (50 μg) emulsified with IFA can stimulate priming of pGPC3-specific CTLs *in vivo* [10]. To analyze the immunogenic potential of the pGPC3-liposome vaccine, A2-Tg mice were immunized with pGPC3(A2)-liposome containing CpG ODN, one of the ligands for toll-like receptor 9 (TLR9) [23], (pGPC3(A2)-liposome/CpG), and the frequency of pGPC3-specific CTLs was evaluated by IFN-γ ELISpot assay. Vaccination with pGPC3(A2)-liposome/CpG induced pGPC3(A2)-specific $CD8^+$ CTLs at a low dose (1 μg) that was 1/50 the amount of peptide used in conventional IFA-emulsified vaccine (Fig. 1B). In contrast, when A2-Tg mice were immunized with IFA-emulsified pGPC3(A2) vaccine containing an equal dose of pGPC3 (1 μg), no pGPC3(A2)-specific $CD8^+$ CTLs were detected (Fig. 1C).

Next, B6 mice were immunized with pGPC3(B6)-liposome containing CpG ODN (pGPC3(B6)-liposome/CpG) or IFA-emulsified pGPC3(B6). The results showed that vaccination with both pGPC3(B6)-liposome/CpG and IFA-emulsified pGPC3(B6) induced pGPC3(B6)-specific $CD8^+$ CTLs at a low (10 μg) or high (50 μg) dose (Fig. 1E, F, G). However, the frequency of pGPC3(B6)-specific $CD8^+$ splenocytes in mice immunized with pGPC3(B6)-liposome/CpG tended to be higher than that in mice treated with IFA-emulsified pGPC3(B6) vaccine (Fig. 1E, F).

The results suggested that pGPC3-liposome elicits peptide-specific CTLs *in vivo* and liposome-coupled peptide vaccine is more effective than IFA-emulsified vaccine for CTL induction.

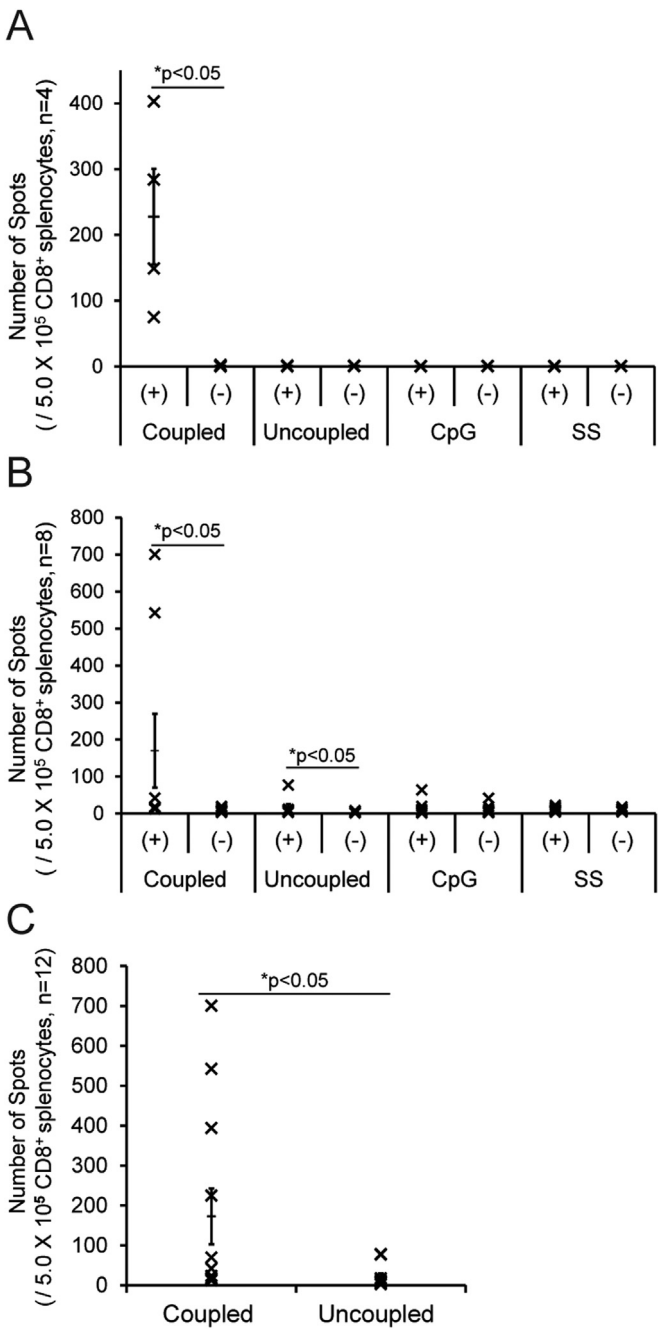

Fig. 2. Coupling of peptides to liposomes is crucial for effective induction of peptide-specific CTLs. (A) A2-Tg mice (n = 8) were immunized with pGPC3(A2) liposome containing CpG as shown in Fig. 1A. $CD8^+$ splenocytes were cocultured with peptide-pulsed (+) or non-pulsed (−) RMA-S-HHD, and the frequencies of IFN-γ-producing cells were evaluated by IFN-γ ELISpot assay. pGPC3(A2)-liposome/CpG: pGPC3(A2) coupled-liposome containing CpG ODN, pGPC3(A2): mixture of pGPC3(A2), liposome, and CpG ODN, liposome/CpG: mixture of liposome and CpG ODN. (B) B6 mice (n = 8) were immunized with pGPC3(B6) liposome containing CpG as shown in Fig. 1A. $CD8^+$ splenocytes were cocultured with peptide-pulsed (+) or non-pulsed (−) RMA-S, and the frequencies of IFN-γ-producing cells were evaluated. (C) B6 mice were immunized with pGPC3(B6)-liposome containing CpG or mixture of pGPC3(B6), liposome, and CpG as indicated in Fig. 1A. $CD8^+$ splenocytes were cocultured with pGPC3(B6)-pulsed RMA-S, and the frequencies of IFN-γ-producing cells were evaluated. (B, C) pGPC3(B6)-liposome/CpG: pGPC3(B6) coupled-liposome containing CpG ODN, pGPC3(B6): mixture of pGPC3(B6), liposome, and CpG ODN, liposome/CpG: mixture of liposome and CpG ODN. $^*p < 0.05$.

3.2. Coupling of the peptides to liposomes is important for CTL induction

Next, we investigated whether coupling of the peptides to liposomes is required for effective CTL induction. A2-Tg mice were immunized with a mixture of pGPC3(A2)/liposome/CpG ODN (uncoupled pGPC3(A2) liposome vaccine) or with pGPC3(A2)-liposome/CpG (coupled pGPC3(A2) liposome vaccine), and the frequencies of pGPC3-specific CTLs were evaluated. The coupled pGPC3(A2) liposome vaccine effectively induced pGPC3(A2)-specific $CD8^+$ CTLs in the spleen (Fig. 2A). In contrast, when the mice were immunized with uncoupled pGPC3(A2) liposome vaccine, pGPC3-specific $CD8^+$ CTLs were not detected (Fig. 2A).

Next, B6 mice were immunized with a mixture of pGPC3 (B6)/liposome/CpG ODN (uncoupled pGPC3(B6) liposome vaccine) or with pGPC3(B6)-liposome/CpG (coupled pGPC3(B6) liposome vaccine). Vaccination with both uncoupled and coupled pGPC3(B6) liposome vaccine induced pGPC3(B6)-specific $CD8^+$ CTLs (Fig. 2B). However, the frequency of pGPC3(B6)-specific $CD8^+$ CTLs in mice injected with the coupled vaccine was higher than that in mice that had received the uncoupled vaccine (Fig. 2C).

These results suggested that the coupling of the peptides to liposomes is essential for effective induction of pGPC3-specific CTLs *in vivo*.

3.3. Liposome-coupled pGPC3 inhibits GPC3-expressing tumor growth in vivo

To investigate the antitumor effect of the pGPC3-liposome vaccine, A2-tg mice were immunized and subcutaneously injected with RMA-HHD-GPC3 cells (expressing murine GPC3) according to the schedule in Fig. 3A. The antitumor effect was evaluated by measuring tumor growth. The tumor area in mice immunized with pGPC3(A2)-liposome/CpG was smaller than in the other groups, and complete regression was observed in one of the mice treated with pGPC3(A2)-liposome/CpG (Fig. 3B,C). These results suggested that pGPC3-liposome vaccine exerts antitumor effect in GPC3-expressing tumor model.

4. Discussion

Multiple clinical trials have used IFA-emulsified peptide vaccination to induce TAA-specific T cell responses in cancer patients. Recently, it has been reported that IFA-emulsified peptide vaccination causes CTL hypo-responsiveness in a murine model [15]. Therefore, new vaccines without IFA are desired.

Liposomal vaccines have been prepared by entrapment of antigens within the aqueous lumen of liposomes, which, besides their role as antigen carrier, are known to act as adjuvants. In a preliminary study, we tried to encapsulate pGPC3 within liposomes; however, no encapsulated pGPC3 could be detected (data not shown), suggesting that the peptide might be too short to be retained within the liposomes. Therefore, we aimed to develop an alternative preparation method for the liposomal peptide vaccine. We previously reported coupling of antigens and short peptides to the surfaces of liposomes as an alternative technique for vaccine preparation [17,22,24]. Furthermore, we showed that liposome-coupled peptides derived from the model antigen OVA inhibited the growth of OVA-expressing tumor [18]. However, the immunological effects of true TAA peptide-coupled liposome have not been investigated to date.

We showed that pGPC3-specific CTLs were effectively induced in mice immunized with pGPC3-liposome vaccine. Moreover, induction was obtained with a lower dose of peptide than that used in conventional IFA-emulsified peptide vaccine (Fig. 1). CpG ODN acts

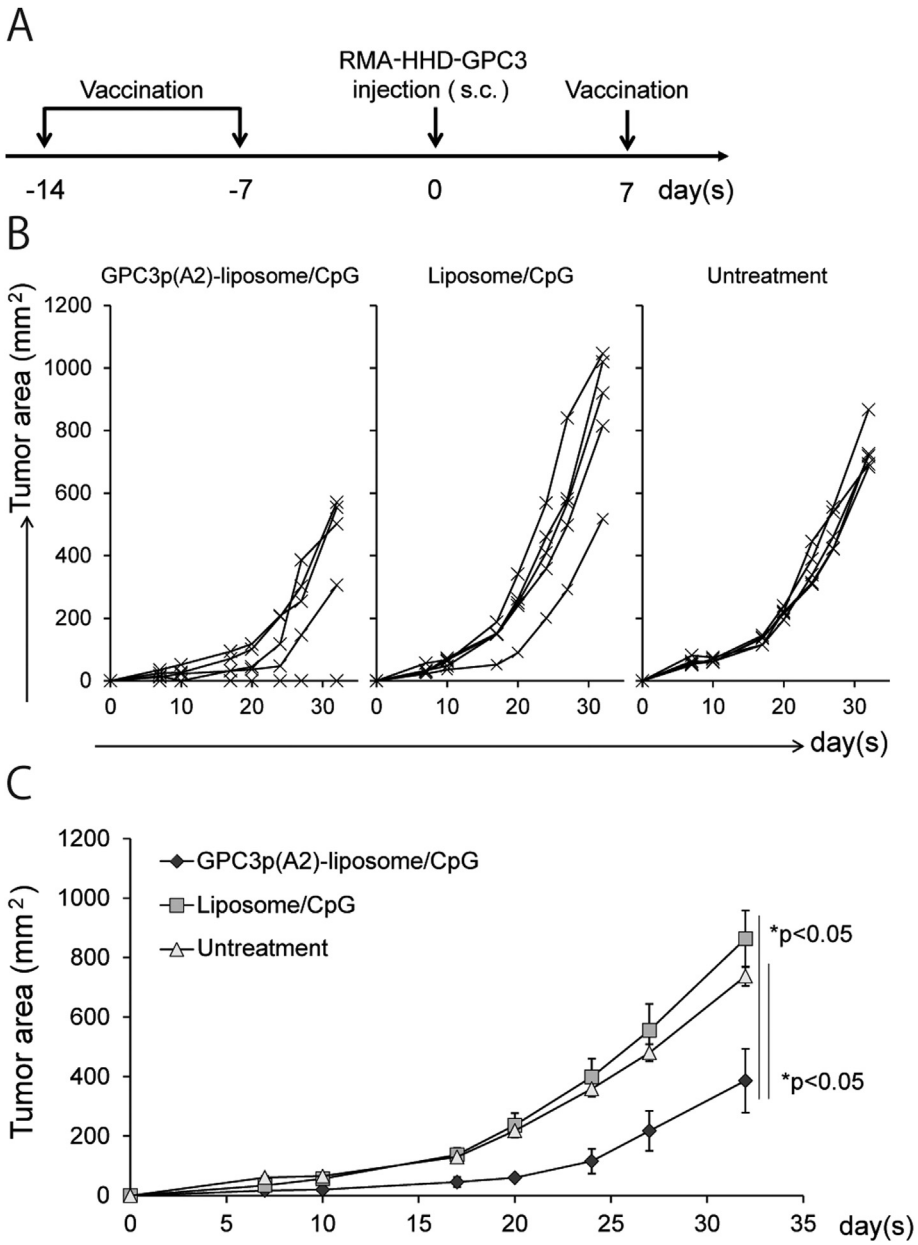

Fig. 3. Vaccination with liposome-coupled pGPC3 inhibits GPC3-expressing tumor growth. (A) Experimental setup for analyzing the *in vivo* antitumor effect. At days −14 and −7, pGPC3 coupled to liposomes containing CpG ODN or a mixture of pGPC3(A2), liposome, and CpG ODN was administrated. RNA-HHD-GPC3 cells were injected at day 0. After 7 days, vaccination was repeated. (B, C) Individual (B) and average (C) tumor areas. Tumor area was calculated according to following formula: (tumor area) = (length of major tumor axis) × (length of minor tumor axis).

as an effective immune adjuvant when co-administered with a CTL epitope peptide by activating antigen-presenting cells, thereby promoting cell-mediated immune responses (Th1 responses) [25]. Indeed, we observed that pGPC3-liposome vaccination with CpG ODN elicited peptide-specific CTLs *in vivo* more effectively than that without CpG ODN (data not shown, Fig. 1). However, despite the presence of CpG ODN, the uncoupled pGPC3/liposome/CpG mixture elicited pGPC3-specific CTLs less effectively than the coupled pGPC3-liposome/CpG vaccine (Fig. 2). These observations suggested that coupling of the peptides to liposomes is essential for effective induction of pGPC3-specific CTLs.

Finally, we demonstrated that liposome-coupled pGPC3 vaccination inhibited GPC3-expressing tumor growth *in vivo* (Fig. 3). It has been recently reported that a "long" peptide vaccine containing both MHC class I and class II epitopes leads to more effective tumor rejection than a "short" peptide containing either an MHC class I or an MHC class II-related epitope [26,27]. It is conceivable that liposome-coupled GPC3-derived long peptide vaccine containing both MHC class I and class II-restricted epitopes has the potential for more effective antitumor effects.

In conclusion, we developed a surface-coupled liposomal pGPC3 vaccine and showed that intradermal administration of this vaccine effectively elicits pGPC3-specific CTLs and exerts antitumor effects against GPC3-expressing tumor *in vivo* in mice. Therefore, liposome-coupled pGPC3 may become a new therapeutic option for HCC as an alternative to IFA-based vaccines and, more generally, surface-coupled TAA peptides may have promising potential in cancer therapy.

Conflicting financial interest

Tetsuya Nakatsura is a scientific advisor to Ono Pharmaceutical Co., Ltd., and is supported by funding from this company. The other authors have no potential conflicts of interest to declare.

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